STUDIES ON THE PHARMACOKINETICS OF BARBITURATES, DIPHENYLHYDANTOIN AND PENTAZOCINE IN MAN WITH SPECIAL REFERENCE TO BLOOD CELL AND PLASMA PROTEIN BINDING

By
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Uppsala 1975

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This thesis is based on the following papers:

I. Ehrnebo, M., Agurell, S., Jalling, B., Boréus, L.O.:

Age differences in drug binding by plasma proteins: Studies on human foetuses, neonates and adults.

Eur. J. Clin. Pharmacol. 3, 189 - 193 (1971).

II. Ehrnebo, M., Agurell, S., Boréus, L.O.:

Gas chromatographic determination of therapeutic levels of amobarbital and pentobarbital in plasma.

Eur. J. Clin. Pharmacol. 4, 191 - 195 (1972).

III. Ehrnebo, M., Agurell, S., Boréus, L.O., Gordon, E., Lönroth, U.:

Pentazocine binding to blood cells and plasma proteins.

Clin. Pharmacol. Ther. 16, 424 - 429 (1974).

IV. Agurell, S., Boréus, L.O., Gordon, E., Lindgren, J.E., Ehrnebo, M. and Lönroth, U.:

Plasma and cerebrospinal fluid concentrations of pentazocine in patients: assay by mass fragmentography.

J. Pharm. Pharmacol. 26, 1 - 8 (1974).

V. Ehrnebo, M.:

Pharmacokinetics and distribution properties of pentobarbital in humans following oral and intravenous administration.

J. Pharm. Sci. 63, 1114 - 1118 (1974).

VI. Ehrnebo, M. and Odar-Cederlöf, I.:

The binding of amobarbital, pentobarbital and diphenylhydantoin to blood cells and plasma proteins in healthy volunteers and uremic patients.

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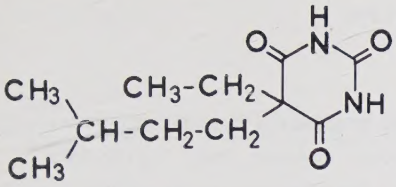
VII. Ehrnebo, M. and Odar-Cederlöf, I.:

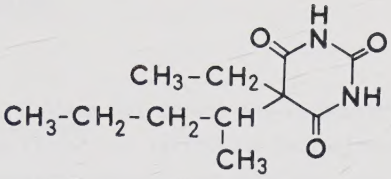
On the localization of pentobarbital and diphenylhydantoin to blood cells: The effect of salicylic acid, temperature and total drug concentration.

Submitted to Eur. J. Clin. Pharmacol.

These papers will be referred to in the text by their Roman numerals.



Amobarbital



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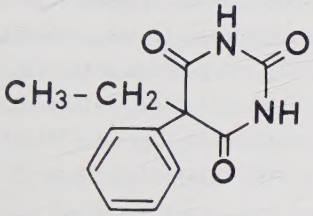

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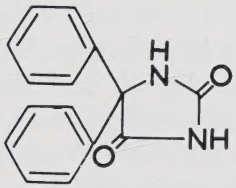
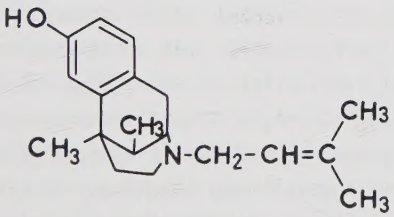


Diphenylhydantoin



Pentazocine

Scheme 1 Chemical structure of studied compounds

INTRODUCTION

Barbiturates are frequently used in clinical practice and their general pharmacological properties are fairly well known. However, very little data have been available on the pharmacokinetics of these drugs in man following therapeutic dosage, probably owing to lack of sensitive analytical procedures for the assay of the drugs in plasma. Recently, Breimer (1974) reported the biological half-lives and biopharmaceutical data of six barbituric acid derivatives; butethal, cyclobarbital, heptabarbital, hexobarbital, methohexital and butylvinal (non-proprietary names according to Merck Index, eighth edition). Half-lives have likewise been reported for amobarbital (Balasubramaniam et al., 1970; Kadar et al., 1973; Krauer et al., 1973) and pentobarbital (Smith et al., 1973), also at therapeutic dosage and by use of analytical methods specific for the parent drug in the plasma of the human adult. The results of Smith et al. (1973) deviate to some extent from the data reported in paper V, as will be discussed later.

The aim of the present study was to develop a sensitive analytical technique for amobarbital and pentobarbital in plasma samples and to study more closely the pharmacokinetics of pentobarbital in man. The plasma protein binding of phenobarbital, amobarbital and pentobarbital and the structurally related compound diphenylhydantoin was taken into special consideration not only in healthy subjects but also in some selected patient groups. However, very few binding studies have taken the blood cells into consideration, although these are the main constituents of human blood. Consequently, the distribution to the blood cells was also investigated for the acidic drugs amobarbital, pentobarbital and diphenylhydantoin and for the basic drug pentazocine, which was also investigated in terms of a preliminary pharmacokinetic model. Finally, some situations which might influence the partition of drugs between blood cells and plasma were also taken into consideration.

Determination of drug binding to plasma proteins

Many different techniques for the measurement of binding of small molecules to proteins are found in the literature, as has been discussed by Meyer and Guttman (1968) and others. The techniques used can be divided into two major groups, non-spectroscopic and spectroscopic methods. The latter, which include the techniques of nuclear magnetic resonance (NMR), electron spin resonance (ESR), fluorescence spectroscopy, circular dichroism and optical rotatory dispersion, are less suitable, however, for the quantitative determination of drug binding to unpurified protein solutions, but can give valuable information about the forces involved in protein-drug interactions. The non-spectroscopic methods comprise the commonly used techniques of equilibrium dialysis, ultracentrifugation, ultrafiltration and frontal analysis chromatography (Copper and Wood, 1968). Of these, the equilibrium dialysis technique was chosen, since it allows the use of small samples and has high reproducibility, as will be considered below.

The equilibrium dialysis technique with the use of cell chambers for 6 - 10 ml samples has been described earlier (Patel and Foss, 1964; Elofsson et al., 1970). In the present studies (I, III, VI, VII), this volume was reduced, so that binding could be estimated on 500 µl samples of plasma. It was possible to reduce the size of the chambers even further, allowing convenient binding experiments on 100 µl samples, as exemplified in Table 1. This makes it possible to perform binding experiments also on capillary blood samples, which is of great value in binding studies on, for example, human neonates.

The reproducibility of the present equilibrium dialysis technique proved to be satisfactory. This is demonstrated in Table 2 which shows the estimated analytical error with data from diphenylhydantoin binding in plasma (VI).

The technique includes the following procedure: 500 µl of plasma is introduced into one of the two chambers whilst labelled drug in buffer solution is introduced into the chamber at the other side of the semi-permeable membrane. The cell is equilibrated at 37.0° C in a water bath and after 5 - 6 hours, samples from each side are withdrawn and the concentration of drug is assayed by liquid scintillation counting. The equilibration time proved to be sufficient for the drugs in the present study, however, other substances, for example the cannabinoids, may need prolonged rocking time (Widman et al., 1974).

Table 1. Comparison between equilibrium dialysis cells for 500 µl plasma and for 100 µl plasma.[a] In vitro binding of ^{14}C-salicylic acid to the same neonatal umbilical cord plasma at a plasma concentration of 160 µg/ml (37.0° C)

	Cells for 500 µl plasma samples [b]	Cells for 100 µl plasma samples [b]
Number of experiments	7	6
Average per cent bound	78.3	77.1
S.D. of average	0.3	0.7

[a] Unpublished data of M. Ehrnebo, S. Agurell, B. Jalling and L.O. Boréus

[b] 50 µl aliquots withdrawn from each side after dialysis

Table 2. Estimated analytical error (σ) in the equilibrium dialysis technique.[a] Plasma protein binding experiments with ^{14}C-diphenylhydantoin in healthy subjects and uremic patients (VI).

	Healthy subjects	Uremic patients
Number of subjects (n)	13	7
Average per cent bound	88.1	74.6
S.D. of average	0.9	2.0
Average analytical error$_a$(σ) in per cent bound[a]	0.2	0.3

[a] Estimated as $\sigma = (\Sigma d^2 / 2n)^{0.5}$, where d is the difference in per cent bound between duplicate determinations on the same plasma sample

At equilibration, the concentration of drug in the buffer phase (C_{BU}) is the same as the plasma water concentration and the per cent bound (100 f_p) can hence be calculated (I, III) as

$$100\ f_p = 100\ (C_p - C_{BU})\ /\ C_p \qquad \text{(Eq. 1)}$$

where C_p is the corresponding plasma concentration of drug. This equation does not compensate for the Donnan effect of the protein solution (Keen, 1966). This effect, however, is difficult to assess both theoretically and practically and, in any case, has little influence except possibly for charged drugs with low protein binding (less than approx. 10 per cent bound in plasma). Other minor effects in the binding experiments such as the osmolality of the plasma solution and the slight increase in plasma pH during storage (I) have not been taken into account.

Determination of drug distribution to blood cells

These in vitro experiments were carried out both on human whole blood, and on a blood cell suspension (BCS) prepared according to McArthur et al. (1971) as follows: The plasma of the whole blood was separated by centrifugation and the remaining blood cell fraction was washed 6 times with approx. the same volume of isotonic phosphate buffer pH 7.35. Finally, the volume was adjusted with buffer solution to that of the initial sample. The suspension thus consisted mainly of washed erythrocytes suspended in an isotonic buffer system with the same hematocrit value as the initial whole blood sample.

The distribution of drug to blood cells, plasma proteins and plasma water of whole blood, and to blood cells and aqueous phase of the BCS was determined by equilibrium dialysis as well as by incubation experiments. In the dialysis experiments, the same equipment as described in the previous section was used. Whole blood or BCS (500 µl) was introduced into the cell on one side of the membrane and buffer solution (500 µl) with labelled drug on the other side. The cell was equilibrated at 37.0° C for 6 to 7 hours and aliquots were withdrawn from both sides for the determination of drug concentrations by liquid scintillation counting. If the degree of plasma protein binding in the whole blood is known from an experiment on the separated plasma, and the hematocrit value, it is possible to calculate the distribution of drug to the plasma water, plasma proteins and blood cells of the whole blood (III).

Similarly, the distribution of drug to the blood cells in the BCS can be determined by use of drug concentration values from both chamber halves and the hematocrit value (III). In the incubation experiments, the drug concentrations were determined in the whole blood or BCS after incubation and thereafter in the supernatant after centrifugation. In a similar way, the distribution to the various constituents of whole blood or BCS could be determined.

Gas chromatographic determination of amobarbital and pentobarbital

Spectrophotometry has been applied for the determination of amobarbital and pentobarbital in biological samples in earlier investigations on the fate of these drugs in humans (Brodie et al., 1953; Swagdis and Flanagan, 1964; Sjögren et al., 1965). However, these methods lack in specificity and sensitivity, and are in general inappropriate for the determination of barbiturates following therapeutic dosage. Gas chromatography has allowed the development of assay methods for amobarbital and pentobarbital suitable for pharmacokinetic studies. One problem, however, in the estimation of barbiturates is the tendency of the substances to adsorb to the column. This can be eliminated by "on column" N,N-dimethylation with trimethylanilinium hydroxide, introduced by Brochmann-Hanssen and Oke (1969) and this technique was used in the present studies (II, V). The reagent has also been used in the analysis of diphenylhydantoin (Berlin et al., 1972) and phenobarbital (Kupferberg, 1970; Kållberg et al., 1971). Adsorption to the column material can also be prevented by the addition of formic acid to the carrier gas (Welton, 1970; Williams et al., 1973). Other contributors to the amobarbital (Balasubramaniam et al., 1969; Inaba and Kalow, 1972) and pentobarbital (Smith et al., 1973) analysis did not use any special procedures to reduce a possible adsorbtion to the column. Low levels of amobarbital and pentobarbital in human plasma have also been analyzed with the aid of gas chromatography - mass spectrometry (Draffan et al., 1973), where the barbiturates are recorded as their N,N-dimethyl derivatives. The radioimmunoassay technique for the determination of pentobarbital in rat plasma (Flynn and Spector, 1972) is also sensitive, but does not distinguish between parent drug and metabolites. Recently, improvements in sensitivity in the gas chromatographic assay of barbiturates have been reported using the nitrogen selective flame ionization detector (Breimer and Van Rossum, 1974; Riedmann, 1974), which also further reduces the plasma "ghost peaks". It

requires, however, precise tuning and more careful handling than the usual flame ionization detector.

At the start of the gas chromatographic study (II) there was only one specific method available for the determination of amobarbital (Balasubramaniam et al., 1969), but it was laborious and had poor precision at low concentrations (II). No convenient method was available for the estimation of pentobarbital at therapeutic levels in plasma. Briefly, the method included the following procedure: To 500 µl of plasma was added 25 µl of internal standard (40 µg/ml of vinbarbital or secobarbital sodium in methanol for the analysis of amobarbital or pentobarbital, respectively). The sample was buffered to pH 5.5 and extracted with 1.0 ml of diethylether. The barbiturates were re-extracted into aqueous trimethylanilinium hydroxide and recorded as their N,N-dimethyl derivatives in the gas chromatograph (Fig. 1). The yield in the extraction steps was approx. 100 % for amobarbital and pentobarbital. Since vinbarbital and secobarbital sodium also are commercially available in different pharmaceutical preparations and appear in the plasma at similar low levels (Breimer, 1974; Sjögren et al., 1965), the method could be useful in biopharmaceutic studies on these drugs, too. The principle with the re-extraction into a small volume of aqueous methylating agent, which provides a rapid, convenient concentration step, was first reported by MacGee (1970), who used trimethylammonium hydroxide in the determination of diphenylhydantoin in plasma.

Mass fragmentographic determination of pentazocine

Analytical methods (Beckett et al., 1970 b; Borg and Mikaelsson, 1970) for the estimation of pentazocine available at the start of the investigations did not allow the repeated determination of this drug in the cerebrospinal fluid (CSF) following therapeutic dosage. Measurement of these low levels (less than 20 ng/ml) by the sensitive techniques of mass fragmentography was therefore investigated. Recently, the electron-capture detection of small amounts of pentazocine in human plasma by means of alkylation with pentafluorobenzyl bromide has been reported (Brötell et al., 1973). The nitrogen detector may also become of value in the assay of pentazocine (James and Waring, 1973).

In the present method, pentazocine and the internal standard cyclazocine in a 1.0 ml sample of plasma or CSF are extracted at pH 10.5 with 6.0 ml

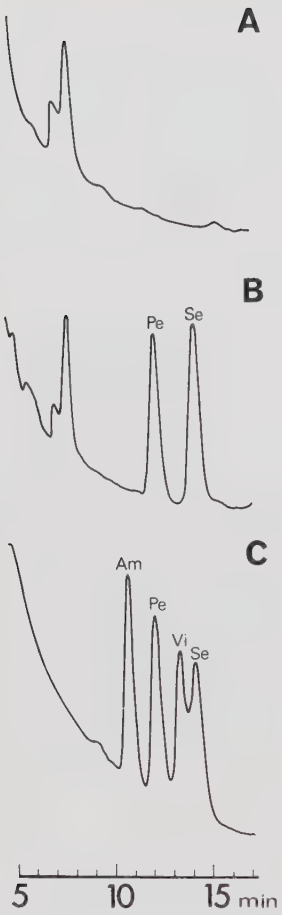


Fig. 1 -(A) Gas chromatogram of control plasma. - (B) Gas chromatogram of patients plasma. Pe = methylated pentobarbital. Se = methylated secobarbital (internal standard). - (C) Gas chromatogram of the four barbiturates as methylated derivatives. Am = methylated amobarbital. Vi = methylated vinbarbital. (II).

benzene. The recovery in this step, determined with ^{3}H-pentazocine, was 92 per cent (plasma) or 94 per cent (CSF). 5.0 ml of the benzene phase was evaporated under a stream of nitrogen and the residue dissolved in 0.1 ml of methanol and further evaporated to 10 - 20 µl. An aliquot of this sample was analysed by mass fragmentography (IV).

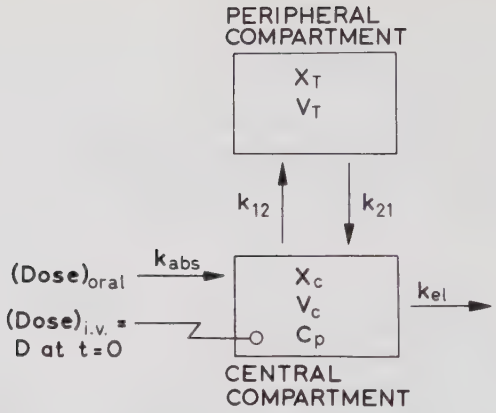
Patient groups

In addition to the healthy subject groups participating in studies I to III and V to VII, some selected patient groups were also examined from different points of view. These consisted of newborns with hyperbilirubinemia (I), patients admitted to the hospital for neurosurgical intervention (III, IV) and uremic patients (VI).

The pharmacokinetic model

Most drugs will show at least a biphasic time - plasma concentration relationship in man following the rapid intravenous injection of the drug, i.e. the two compartment open body model may be applied (Riegelman et al., 1968 a). The model (Scheme 2) consists of a central compartment, sometimes misleadingly called the plasma or blood compartment, which is instantaneously accessible to the drug, and the peripheral compartment, which is more slowly accessible to the drug. It is assumed that elimination takes place from the central compartment, and that the rate constant for elimination from it (k_{el} or k_e) as well as the distribution rate constants (k_{12} and k_{21}) and the absorbtion rate constant (k_{abs}) is of first order. The solution of these rate constants from the bi-exponential function, regarding the I.V. bolus dose, or from the three-exponential function, regarding the oral dose, has been described earlier (cf. Riegelman et al., 1968 a; Wagner et al., 1971). The commonly used entities for the distribution volume of the central compartment (V_C or V_1), the volume of the peripheral compartment (V_T or V_2) at steady state and the total volume of central and peripheral compartment, $(Vd)_\beta$, during the disposition phase (β-phase), all with reference to plasma or whole blood concentrations, have also been derived previously (Wagner et al., 1968; Gibaldi et al., 1969), and these parameters are summed-up in paper V. The various rate constants of this model were evaluated with the aid of a digital computer (IBM 360, revised BMDX 85 program) by a method for nonlinear least-squares fit.

The central compartment, however, is not solely a blood compartment in most cases, but also includes the associated fluid, i.e. tissues, which are instantaneously accessible to the drug. This could be considered to consist of a highly perfused tissue group such as the heart, kidney, lung and hepato-portal system. Since the uptake of the drug by the highly perfused tissue



Scheme 2. The two compartment open body model. X_T = amount drug in peripheral compartment. V_T = volume of peripheral compartment at steady state. X_C = amount drug in central compartment. V_C = volume of central compartment. C_p = plasma concentration.

group is usually rapid, it can be conceived mathematically to be a part of the central compartment (Riegelman et al., 1968 a). Since studies were undertaken on drug binding to plasma proteins and blood cells in vitro (I, III) it was found important to calculate more exactly the fractional distribution of a drug to the various components of central compartment (V). The fractions of amount in the central compartment distributed to plasma water (F_{PW}), plasma proteins (F_{PP}), blood cells (F_{BC}) and associated fluid (F_{AF}) were defined as:

$$F_{PW} = a_{PW} / X_C \qquad \text{(Eq. 2)}$$

$$F_{PP} = a_{PP} / X_C \qquad \text{(Eq. 3)}$$

$$F_{BC} = a_{BC} / X_C \qquad \text{(Eq. 4)}$$

$$F_{AF} = a_{AF} / X_C \qquad \text{(Eq. 5)}$$

where a_{PW}, a_{PP}, a_{BC} and a_{AF} are the corresponding amounts of drug distributed

to plasma water, plasma proteins, blood cells and associated fluid, respectively, and X_C the total amount of drug in the central compartment. Moreover, the drug binding to plasma proteins and blood cells was also taken into account regarding the distribution volumes of unbound drug in the two compartment model (V). The volume of distribution of associated fluid and plasma water (V_Pu) (Garrett and Lambert, 1973) with reference to the concentration of drug in plasma water (C_{PW}) was calculated as:

$$V_Pu = (a_{AF} + a_{PW}) / C_{PW} = V_C (F_{AF} + F_{PW}) / (1 - f_P) \qquad \text{(Eq. 6)}$$

The corresponding distribution volume of the peripheral compartment during the β-phase relative to the plasma water concentrations, $(V_Tu)_\beta$, was calculated as:

$$(V_Tu)_\beta = \left[(Vd)_\beta - V_C \right] / (1 - f_P) \qquad \text{(Eq. 7)}$$

PLASMA PROTEIN BINDING OF BARBITURATES, DIPHENYLHYDANTOIN AND PENTAZOCINE

Plasma protein binding in healthy subjects

It has earlier been demonstrated that there are only small inter-individual differences in the per cent of bound diphenylhydantoin in the plasma of healthy adults (Ganshorn and Kurz, 1968, Lunde et al., 1970). This was also found for all drugs in the present studies (I, III, VI) within the healthy subject group. Disregarding these narrow ranges, however, it is interesting to note that there is an individual correlation between the binding of pentobarbital versus that of amobarbital or diphenylhydantoin (VI). For example, an individual with a low binding degree of pentobarbital in plasma has also a low binding degree of diphenylhydantoin (Fig. 2). The individual correlation in the fractional binding of these three drugs suggests a similarity in the main binding sites of these drugs. This is in accordance with the work by Nilsson et al. (1973), who found indications that some acidic drugs compete for the same binding sites on purified human serum albumin.

The fractional binding of pentazocine to plasma proteins is essentially independent of the concentration of pentazocine at levels well above therapeutic concentrations (III). The same is true for pentobarbital up to 62 µg/ml, although further increase in total concentration probably would have caused decreased fraction bound (Brånstad et al., 1972). Salicylic acid showed decreased fraction bound with increasing plasma concentration within the therapeutic range (VII). The same has been reported for diphenylhydantoin (Lunde et al., 1970) in the range 0 - 300 µg/ml plasma.

The dependence of age and hyperbilirubinemia

An age dependent variation in plasma protein binding of drugs was first reported by Behrman and Battaglia (1967), Ganshorn and Kurz (1968) and Sereni et al. (1968), who found decreased drug binding in the neonatal plasma compared to that of the adult. In the present study (I), the foetal plasma and plasma from neonates with hyperbilirubinemia were also taken into consideration. The percentage bound in plasma was investigated for phenobarbital, diphenylhydantoin and three different types of penicillin. For all the drugs, the degree of

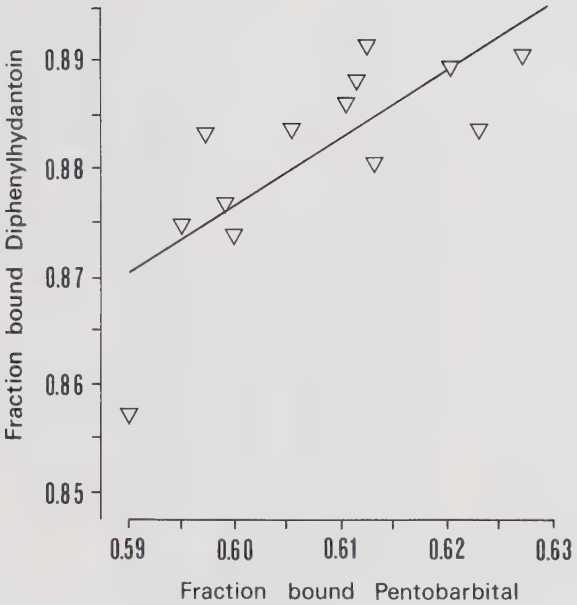


Fig. 2 The ability of individual plasma samples (healthy subjects) to bind diphenylhydantoin against ability to bind pentobarbital. The determinations were made on two separate occasions on the same individual plasma sample with only one drug present. (VI).

binding increased with age (Fig. 3); for example, the mean value for phenobarbital was 24 per cent bound in the foetal plasma, 36 per cent in the neonatal plasma and 51 per cent in the adult plasma. This can partly be explained on differences in protein concentrations (I), but qualitative differences in drug-binding proteins at various ages may also be of importance as well as different levels of interacting endogenous substances. Moreover, hyperbilirubinemia in the newborn caused a lower degree of binding of all five drugs as compared with newborns with normal levels of bilirubin in spite of the similarity in albumin and total protein concentrations (I). It has been suggested (cf. Rane et al., 1971), that this could be due to an interaction between bilirubin and drug for binding sites on the albumin molecule. However, in the

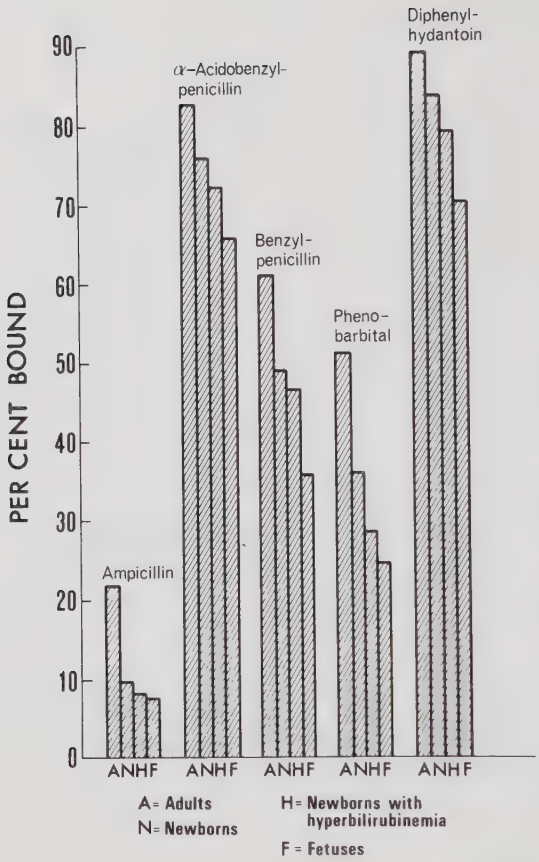


Fig. 3 Drug-plasma binding at different ages: Mean percentage of drug bound by plasma from the four groups studied. (I).

present study, no correlation was found between bilirubin level and the proportion of unbound drug.

The difference in plasma protein binding between adults and newborns has also been examined by Pruitt and Dayton (1971) and Chignell et al. (1971) with results in accord with the present study (I).

In the healthy subject group, there are only small inter-individual differences in the per cent bound in plasma (III). However, in patients admitted to the hospital for neurosurgical intervention there is a larger variation in pentazocine binding than in the normal control subjects (Fig. 4). Anton and Corey (1971) have reported altered plasma protein binding of sulfadimethoxine in hospitalized patients with various diagnoses other than uremia, such as arthritis, cancer, hepatitis. These pronounced variations in the degree of binding may be due to other drugs, to differences in circulating endogenous metabolites, to altered protein structure or to changes in protein concentration. It should be pointed out, however, that the neurosurgical patients (III) all had total plasma protein concentrations within normal range (65 to 80 mg/ml).

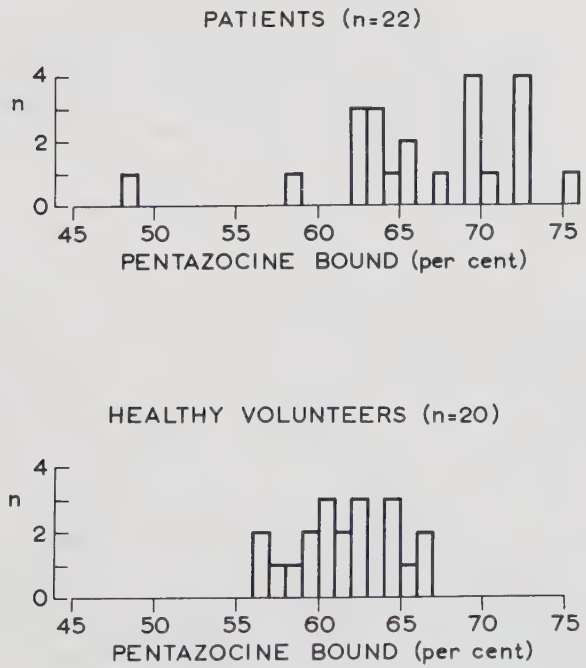


Fig. 4 The distribution of plasma protein binding values of patients admitted to the hospital for neurosurgical intervention (above) and normal subjects (below). (III).

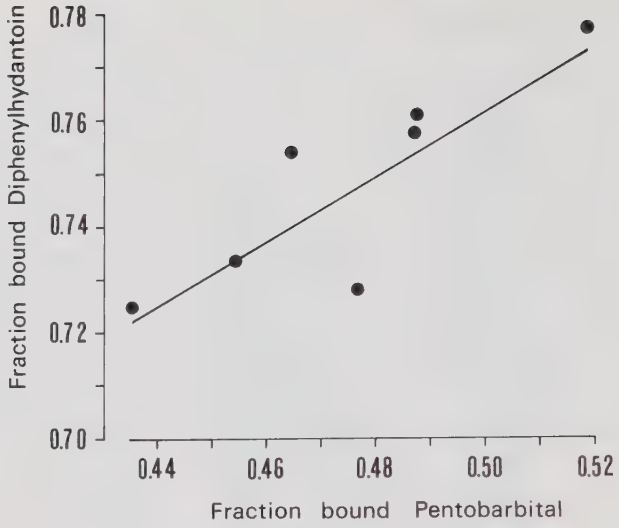


Fig. 5 The ability of individual plasma samples (uremic patients) to bind diphenylhydantoin against ability to bind pentobarbital. The determinations were made on two separate occasions on the same individual plasma sample with only one drug present. (VI).

Plasma protein binding in uremic patients

It has been shown, that uremic patients have a decreased plasma protein binding of certain drugs such as diphenylhydantoin (Reidenberg et al., 1971; Shoeman and Azarnoff, 1972) and sulfonamides (Anton and Corey, 1971). Recently, a decreased binding of some acidic drugs, among them thiopental, was reported during acute renal failure (Andreasen, 1973). The present results (VI) on pentobarbital and diphenylhydantoin are in accordance with these findings. Also, the decrease of the bound fraction of pentobarbital or diphenylhydantoin is correlated with the severity of uremia, expressed as serum creatine concentration and hematocrit value. There is also a positive correlation between the fraction bound of the two drugs and the serum albumin concentration in the uremic patients. This might in part be explained as a quantitative difference

in the concentration of binding proteins but it is also possible that the lowered serum albumin concentration values run parallel with other factors influencing the affinity constant of the binding sites in uremia. As within the healthy subject group (Fig. 2) there is a positive correlation within the uremic patients between the degree of plasma protein binding of pentobarbital and diphenylhydantoin suggesting similar changes in the binding sites of these drugs (Fig. 5).

DISTRIBUTION OF BARBITURATES, DIPHENYLHYDANTOIN AND PENTAZOCINE TO BLOOD CELLS

The distribution of drugs to blood cells of healthy subjects

Most studies on drug binding has been undertaken on plasma, serum or purified albumin solutions, but the possible binding of drugs to the human blood cells has received comparatively little attention. The distribution of drug to the human blood cells has been quantitatively estimated for a series of sulfonamides (Witzgall and Boyens, 1964) and for digitoxin (Abshagen et al., 1971). Recently, human erythrocyte uptake of diphenylhydantoin (Borondy et al., 1973; Kurata and Wilkinson, 1974; Hansotia and Keran, 1974) and propranolol (Evans and Shand, 1973) has been taken into consideration. Possible mechanisms for the incorporation are binding to intracellular proteins (Wind et al., 1973) or/and binding to the blood cell membrane (Kwant and Seeman, 1969) or/and merely a distribution in the aqueous phase of the blood cell.

In the present papers (III, VI, VII) all the drugs studied (pentazocine, amobarbital, pentobarbital, diphenylhydantoin, salicylic acid) were found to distribute to the blood cells of whole blood to an appreciable degree, as summarized in Table 3. For example, approximately half the amount of pentazocine present in the whole blood of healthy subjects can be expected to be localized to the blood cells. The distribution of drugs to the blood cells of the whole blood showed little inter-subject variation (Table 3) in the healthy subject group. The rate of equilibration between blood cells and plasma was high and a steady state between drug in blood cells and plasma was obtained within less than 5 minutes, though the current technique did not allow estimates below that time interval. Probably, the steady state is obtained within seconds (Klocke et al., 1972).

The intrinsic capacity of the blood cells to incorporate different drugs is best expressed by the ratio of concentration in blood cells : concentration in surrounding aqueous phase (Table 4). For the whole blood this ratio is designated by R and for the blood cell suspension by R_s. The R_s value was lower than the R value for pentazocine (III), but for amobarbital, pentobarbital and diphenylhydantoin the R_s value was higher than the R value (VI). The difference in R and R_s may depend on the presence of plasma proteins, possibly due to an interaction of the protein molecules with the erythrocyte membrane (VI), but further studies are needed. However, all values of R and

Table 3. Summary of data for the distribution of some drugs to the blood cells in the whole blood (in vitro)

Drug	Paper	Whole blood concentration	Number of subjects	Average hemato-crit	Percentage drug in blood cells [a]
Pentazocine	III	230 ng/ml	3	0.45	48.1
Amobarbital	VI	2 µg/ml	16	0.44	32.8 ± 4.0
Pentobarbital	VI				
healthy subjects		2 µg/ml	17	0.44	33.0 ± 4.0
uremic patients		2 µg/ml	8	0.29	25.1 ± 7.4
Diphenylhydantoin	VI				
healthy subjects		2 µg/ml	10	0.44	24.7 ± 4.0
uremic patients		3 µg/ml	6	0.29	30.2 ± 7.5
Salicylic acid	VII	40 µg/ml	2	0.43	4.7

[a] Mean values (± S.D.) for the per cent of amount in whole blood distributed to the blood cells

R_s for the studied drug exceeded the cell to plasma water concentration ratio of freely diffusible anions or cations (Kintner, 1972). This indicates that the drugs are not only distributed in the aqueous phase of the blood cell, but also bound in or/and to the cell to some extent. As seen in Table 4, this degree of binding was highest for diphenylhydantoin and lowest for salicylic acid.

It has been reported (Borgå et al., 1968) that the plasma protein binding of desmethylimipramine shows considerable species differences. This is also true for amobarbital (Garrett et al., 1974). Furthermore, it is also probable that there exists a species difference in the ratio between concentration in blood cells : concentration in plasma water, since Garrett et al. (1974) have reported this ratio to be 1.0 (± 0.2) for amobarbital in the dog.

Table 4. Summary of data for the distribution ratios of some drugs in whole blood and blood cell suspension (in vitro)

Drug	Paper	Number of subjects	Ratio [a] between concentration in Whole blood and plasma (C_B / C_P)	Blood cells and plasma water (R)	Blood cells of BCS and buffer phase (R_s)
Pentazocine	III	3	1.06	3.05	2.68
Amobarbital	VI	15 - 16	0.835 ± 0.032	1.50 ± 0.15	1.72 ± 0.12
Pentobarbital	VI				
healthy subjects		17	0.827 ± 0.049	1.59 ± 0.17	1.84 ± 0.04
uremic patients		6 - 8	0.950 ± 0.035	1.56 ± 0.09	1.81 ± 0.10
Diphenylhydantoin	VI				
healthy subjects		10 - 13	0.748 ± 0.027	3.55 ± 0.41	4.74 ± 0.47
uremic patients		6	0.984 ± 0.037	3.83 ± 0.28	4.71 ± 0.27
Salicylic acid	VII	2	-	-	1.32

[a] Mean values (± S.D.)

The distribution of pentobarbital and diphenylhydantoin to blood cells of uremic patients

The uremic blood cells carried appreciable proportions of the pentobarbital and diphenylhydantoin present in the whole blood, as seen in Table 3, in spite of the decreased hematocrit value of uremic patients (VI).

As stated earlier, the plasma protein binding of pentobarbital and diphenylhydantoin was significantly decreased in uremic patients as compared to healthy subjects. However, the ratios (R and R_s; Table 4) between concentration in blood cells and concentration in aqueous phase were not significantly different from those in the healthy subjects for the two drugs (VI). Thus, neither the pathological changes in uremic erythrocytes (Erslev, 1969), nor the altered plasma composition in uremia seemed to influence the affinity of the blood cells for the two acidic drugs.

Influence of total concentrations on the distribution of drug to blood cells

As mentioned above, the degree of plasma protein binding was independent of concentration within the therapeutic range for pentazocine (III) and pentobarbital (VII) whereas for salicylic acid (VII) and diphenylhydantoin (Lunde et al., 1970) the binding degree was decreased with increasing concentrations. In the blood cell suspension, the fractional distribution to the blood cells was unaffected by total drug concentration within therapeutic ranges not only for pentazocine (III) and pentobarbital (VII) but also for salicylic acid and diphenylhydantoin (VII). As expected, the distribution to plasma water, plasma proteins and blood cells of whole blood was constant within therapeutic concentrations for pentazocine (III) and pentobarbital (VII). For salicylic acid, the fractional distribution to plasma proteins, blood cells and plasma water depended on the total concentration within the therapeutic range. Thus, in accordance with the decreased plasma protein binding degree and the unaffected distribution to the blood cells, increase in total whole blood concentrations of salicylic acid in vitro caused a shift of the drug from the plasma proteins to the blood cells and plasma water.

Interactions with salicylic acid in the whole blood

It has been shown previously that acidic drugs displace diphenylhydantoin (Lunde et al., 1970) and pentobarbital (Nilsson et al., 1973; Pagnini et al., 1971) from plasma protein binding sites. This is further demonstrated in the present investigation (VII) for diphenylhydantoin and pentobarbital which are displaced by increasing plasma concentrations of salicylic acid. However, it was not possible to displace pentobarbital or diphenylhydantoin from binding sites in/on the blood cells in the BCS with salicylic acid concentrations up to 180 µg/ml in aqueous phase. Thus, in whole blood, the fractions of pentobarbital or diphenylhydantoin displaced from the plasma proteins by salicylic acid in vitro will distribute to the blood cells and plasma water, in accordance with an approx. unchanged blood cell to plasma water concentration ratio (VII).

Whole blood versus plasma analysis: The effect of temperature

From the analytical-chemical point of view, the question arises whether to analyze whole blood or plasma. According to the previous investigation, it might be considered as an analytical extravagance to reject the blood cell fraction and just analyse the plasma aliquot, especially if sample sizes are limited such as in capillary blood sampling. Another approach to the problem, advantageous in the analysis of whole blood, is the temperature effect, exemplified in the following with diphenylhydantoin:

A decrease in temperature from 37° C to 25° C does not significantly alter the distribution of diphenylhydantoin to the blood cells of BCS (VII), i.e. the ratio R_s is the same at both temperatures. However, the fraction of free diphenylhydantoin in plasma is decreased about 39 per cent at room temperature compared with 37° C (Lunde et al., 1970). Thus, if the whole blood concentration is constant, the concentration in plasma will change as the free fraction in plasma changes. For example; if a blood sample containing diphenylhydantoin is withdrawn from the patient at body temperature (37° C), and the blood is allowed to equilibrate at 25° C before separating the plasma, a difference between the in vivo and the in vitro plasma concentration will result. It can be calculated (VII) that this treatment will overstimate the in vivo plasma concentration of this drug with about 7 - 10 per cent. These differences could be even more pronounced if the separation of plasma is undertaken at refrigerator temperatures.

The two compartment open body model

Pentazocine. Pentazocine is eliminated from the body mainly through metabolism; only about 3 - 8 per cent of the dose following oral administration is excreted unchanged in the urine under conditions of controlled acidic urine (Vaughan and Beckett, 1974). Following the i.v. dosage (30 mg), pentazocine levels declined biexponentially (IV) according to the two compartment model (Riegelman et al., 1968 a). The α-phase was completed after approx. 30 min, and the β-phase half-life, estimated from values up to 3 hours, was found to be 134 min. in agreement with previous published data (Berkowitz et al., 1969).

The analogue computed amounts of pentazocine in peripheral ("tissue") compartment was found to reach a maximum mean value at approx. 25 min. (IV). It is interesting to note that Berkowitz et al. (1969) reported that maximum analgesia after intravenous pentazocine injections occurs after about 30 min., suggesting that the target for the pentazocine effect is situated in the peripheral rather than in the central compartment. Moreover, Pittman and Portmann (1974) recently showed that predicted tissue levels of pentazocine in the rhesus monkey were in agreement with the observed cerebral concentrations in biopsy samples.

The present pharmacokinetic model (IV) of pentazocine and its rate constants is to be regarded as a preliminary model, since its values were estimated from average plasma concentrations covering a 3 hour period after administration, and since the present investigation was performed on anaesthetized patients.

Pentobarbital. Pentobarbital is eliminated in man almost entirely through metabolism; less than one per cent of the drug has been reported to be excreted unchanged in the 48 hour urine following intravenous administration (Brodie et al., 1953). Its main metabolite is 5-ethyl-5-(3-hydroxy-1-methylbutyl) barbituric acid and it has been shown that this metabolite occurs in the dog in four different diastereoisomers, since pentobarbital, as normally used, is a racemic mixture of the R- and S-isomers (Palmer et al., 1969; Palmer et al., 1970). In mice, the S (-) isomer was reported to be more potent in both anaesthetic and lethal activity than the R (+) isomer (Waddell and Baggett, 1973). Moreover, the optical antipodes of hexobarbital has been shown to have different plasma half-lives in man (Breimer and van Rossum, 1973). However,

inequalities in the pharmacokinetics of R- and S-pentobarbital has not been taken into account in the present investigation (V).

The plasma concentrations of pentobarbital following the intravenous injection (100 mg) were also found to fit the two compartment model (V). The volume of the central compartment (V_C; Scheme 2), relative to plasma concentrations varied very little between the individuals (27.8 ± 3.3 liters; S.D., n = 7) whereas the volume of the peripheral compartment (V_T) showed greater variation (35.6 ± 12.9 liters; S.D., n = 7) between individuals. Furthermore, V_T showed an increase in volume with an increase in body weight, but no significant increase in V_C with body weight was observed. The increase in V_T with body weight for the lipid soluble pentobarbital is probably due to additional adipose tissue and muscles in the subjects with higher body weights. These relationships will also influence the ratio of amount pentobarbital in peripheral tissues : amount in the central compartment (Fig. 6). This ratio, $(X_T / X_C)_\beta$,

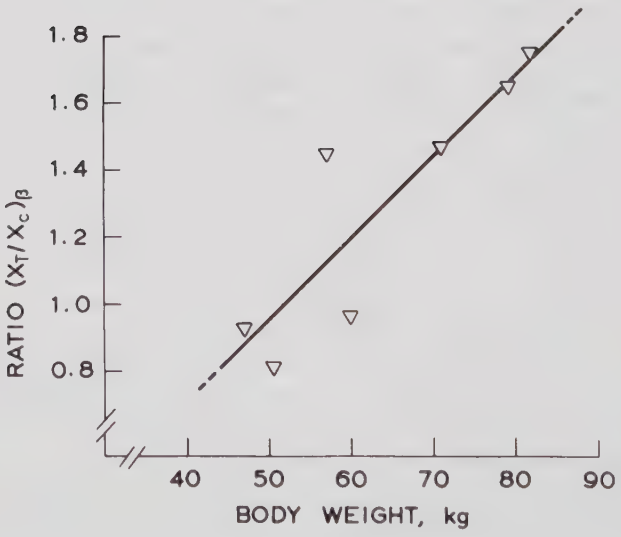


Fig. 6 Ratio of amount in peripheral compartment / amount in central compartment during the β-phase versus body weight in seven healthy volunteers following administration of 100 mg pentobarbital by rapid intravenous injection. (V).

valid during the β-phase, increases with increased body weight, and consequently, subjects with a body weight of about 80 kilograms will have more of pentobarbital in their peripheral tissues than those weighting about 50 kilograms.

The estimated β-phase half-lives (t 1/2) of pentobarbital, reported in the literature or to be published, are listed in Table 5. Regarding therapeutic

Table 5. Half-lives (t 1/2) of pentobarbital at different dosage levels (normal subjects)

Author(s)	Dosage	Half-life (hrs)	Method of analysis
Brodie et al. (1953) and Riegelman et al. (1968 b)	1000 mg i.v.	41	Spectrophotometry
Fazekas et al. (1956)	800 - 1000 mg p.o.	46	Spectrophotometry
Misra et al. (1971)	6 mg/kg p.o.	35.1 ± 6.1 (n = 4)	^{14}C-pentobarbital
Smith et al. (1973)	50 mg i.v.	60.3 (range 43-75; n = 5)	Gas chromatography
Ehrnebo (V)	100 mg i.v.	22.3 ± 4.0 (S.D.; n = 7)	Gas chromatography
	100 mg p.o.	25.1 ± 5.6 (S.D.; n = 7)	
Reidenberg et al.[a]	100 mg p.o.	21 (range 17.5-24; n = 7)[b]	Gas chromatography
Breimer[c]	100 mg p.o. and p.r.	24 (range 18-31)	Gas chromatography

[a] Reidenberg, M.M., Cowenthal, D.T., Briggs, W.: Personal communication

[b] Two other normal subjects had half-lives > 30 hrs

[c] Breimer, D.D.: Personal communication

dosage and specific analytical technique, only one value for the half-life of pentobarbital has been published, namely that of Smith et al. (1973). They reported the half-life to approx. 50 hrs. However, the more precise value (60.3 hrs) calculated from their reported rate constants (V) is shown in Table 5. The reliability of the present β-phase half-life (V) at 100 mg dosage of pentobarbital is further assessed by the preliminary reports of Reidenberg et al. and Breimer (Table 5). Thus, the nearly three times longer β-phase half-life of Smith et al. (1973) must be due to model-dependent pharmacokinetics of a more complex nature. Decreased sensitivity in the assay of pentobarbital at lower concentration offers another explanation, but, unfortunately, their paper includes no details on sensitivity, recovery or precision at low levels of pentobarbital. The present data (V) on rate constants and volume estimates of the two compartment body model showed further deviations from those reported by Smith et al. (1973). Consequently, a more detailed knowledge of pentobarbital pharmacokinetics at different dosage levels will require further examinations.

Intravenous clearance and bioavailability

Pharmacokinetically, pentazocine and pentobarbital represent two different classes of compounds, that with high intravenous clearance and that with low intravenous clearance, respectively. Provided that the drug is eliminated only in the kidney and the liver, the total clearance is the sum of renal and hepatic clearance. The latter may be defined and calculated as (Rowland, 1972):

$$\text{hepatic clearance} = \dot{V}_{BL} (C_{in} - C_{out}) / C_{in} = f_m D/\lambda \int_0^\infty C_p dt \qquad \text{(Eq. 8)}$$

where $\dot{V}_{BL}$ is the blood flow through the liver; C_{in} and C_{out} the concentrations entering and leaving the liver, respectively; f_m the fraction of the dose (D) metabolized in the liver, $\int_0^\infty C_p dt$ the area under the plasma concentration - time curve and λ the ratio between drug concentration in blood : drug concentration in plasma. Since $D/\int_0^\infty C_p dt = k_e \cdot V_1$ or $k_{el} \cdot V_C$, the hepatic clearance of pentazocine can be calculated using the values $f_m = 0.85$, evaluated from urine data following intravenous administration (Beckett et al., 1970), $\lambda = 1.07$ (III) and mean values for k_e and V_1 (IV). The approximate hepatic clearance is then 1.0 liters/min representing about 66 per cent of the liver blood flow which is 1.53 liters/min (Rowland, 1972). The predicted availability (θ) of a completely absorbed oral dose can be estimated as (Rowland, 1972):

$$\theta = \left| - \frac{\text{hepatic clearance}}{\text{liver blood flow}} \right| \qquad \text{(Eq. 9)}$$

and consequently, the availability of completely absorbed oral pentazocine can be expected to be approx. 34 per cent. This is in agreement with the statement of Beckett et al. (1970) that changes from intravenous pentazocine to oral solution or oral tablet requires a dose increase of 2-3 or 3-5 times, respectively, to obtain similar cumulative urinary excretion profiles and similar blood levels. Moreover, the fecal recoveries of unchanged pentazocine after oral administration is less than one per cent of the dose according to the same authors.

Pentobarbital, on the other hand, has low hepatic clearance; a corresponding calculation using pentobarbital data (V) gives the value 0.05 liters/min for the hepatic clearance. This clearance value is only 3 per cent of the liver blood flow, which suggest that the oral availability of completely absorbed pentobarbital should be about 97 per cent (Eq. 9). The average bio-availability following oral administration of pentobarbital (Mebumal, ACO Läkemedel AB, Solna, Sweden) was in agreement with this value (V).

The binding to blood cells and plasma proteins in relation to the pharmacokinetic model

It is usually considered that drug binding to plasma proteins is one factor influencing the distribution of drugs in the body, since only unbound drug is freely diffusible and potentially able to cross biological membranes (Goldstein et al., 1974). At equilibrium, the concentration in the extravascular water will be the same as the unbound concentration in plasma water, i.e., usually lower than the total plasma level. For example, Lund et al. (1972) have shown that there is an agreement between the unbound fraction of diphenylhydantoin in plasma and the concentration in the cerebrospinal fluid under steady-state conditions. Also, in the apparent distribution equilibrium of the β-phase, one would expect a similar relationship. This appeared to be true for pentazocine, as the mean csf / plasma ratio 2-3 hours after intravenous injection approached the value 0.3 - 0.4 for unbound / total pentazocine in plasma (IV).

Recently, attention has been drawn to *in vitro* binding studies and the importance of defining conditions under which certain phenomena would be expected to occur *in vivo* (Gillette, 1973). Regarding pentobarbital, only a minor fraction of the drug in the body is present in the central circulation. As seen in Table 6, only about 5 per cent of the total drug in the central compartment is bound to the plasma proteins, the main fraction (87 per cent) being distributed to the associated fluid. Consequently the interaction of other drugs with plasma protein binding sites will have little influence on pentobarbital distribution in the body (V, VII). The same appears to be true for diphenylhydantoin (VII). The effect of salicylic acid on the rate of elimination of the two drugs is however more difficult to predict, because of the complexity of liver metabolism (Gillette, 1973). Previously, only unbound drug in plasma has been considered to be available for metabolism (Meyer and Guttman, 1968) and thus increased plasma protein binding would decrease drug elimination rate. However, Evans and Shand (1973) have shown that for propranolol, having high hepatic clearance ($\sim$ 1.1 liter/min), the rate of elimination increases with the degree of drug binding in the blood. Thus, binding in the blood has the effect of transfering the drug from the tissues to the liver, thereby accelerating the elimination rate. In this connection the blood cells carrying the drug serve the same function as the plasma proteins, provided that the dissociation of drug from the blood cells is rapid. Since pentazocine is a drug with a hepatic clearance similar to that of propranolol, differences in binding to blood cells and plasma proteins may influence the elimination kinetics of pentazocine.

Ideally, drug distribution volumes should always be given also with reference to plasma water concentrations. These corrected distribution volumes have the most valid physiological significance (Garrett and Lambert, 1973) and allow comparison with body water estimates. For example, the distribution volume of plasma water and associated fluid for pentobarbital relative to plasma water concentration, $V_P{}^u$ (Eq. 6), far exceeded the total body water as did the peripheral volume, $(V_T{}^u)_\beta$ (Eq. 7), indicating extensive tissue binding of pentobarbital in both compartments (V). Also, abnormal binding phenomena in plasma, such as occurs in uremic patients, may be compensated for (Borgå and Odar-Cederlöf, 1974). The increase in apparent volume of distribution with dose in children that had ingested salicylate has been suggested to be partly due to decreased binding of salicylate to plasma proteins (Levy and Yaffe, 1974). The present results *in vitro* showing decreased binding of salicylic acid in human plasma with increasing concentrations (VII) further supports this idea.

Table 6. Fraction of amount in central compartment distributed to plasma water (F_{PW}), plasma proteins (F_{PP}), blood cells (F_{BC}), and associated fluid (F_{AF}) following 100 mg pentobarbital intravenously (V).

Subject	Fraction drug bound in plasma	F_{PW} x 100	F_{PP} x 100	F_{BC} x 100	F_{AF} x 100
M.E.	0.613	2.9	4.5	3.7	88.9
H.K.	0.601	4.1	6.1	5.3	84.5
K.K.	0.600	3.8	5.7	4.1	86.4

Regarding drugs, which are mainly located in the associated fluid of the central compartment, the distribution volume relative to plasma water concentration may approx. be calculated as V_C / (1 - f_P), since the contribution of the fractions F_{PP} and F_{BC} is small (Eq. 6). For pentobarbital, these approximations will overstimate the "true" distribution volume of free drug in the central compartment by about 10 per cent. However, the fractions distributed to blood cells and plasma proteins become more important when drugs with smaller distribution volumes are considered by this approach. In any case, the distribution volumes of unbound drug should always be taken into account when plasma levels are evaluated in patient groups with special binding characteristics such as the uremic patients (VI), the neonates and the neonates with hyperbilirubinemia (I).

CONCLUSIONS

Gas-chromatographic and mass fragmentographic methods are described for the assay of pentobarbital, amobarbital and pentazocine, respectively, in biological samples. Convenient methods were developed for the estimation of drug distribution to blood cells, plasma proteins and plasma water. These techniques were used to study the plasma protein and blood cell binding of some drugs whilst analytical techniques were used to study the pharmacokinetics of pentazocine and pentobarbital. The general conclusions drawn from these studies in humans are:

1. There was an age - dependent variation in the plasma protein binding of phenobarbital and diphenylhydantoin. Plasma from adults bound a higher proportion of the drug than did neonatal plasma, and hyperbilirubinemia in the neonatal period further decreased the binding degree. The percentage bound was further decreased in foetal plasma compared to neonatal plasma.

2. There was a larger variation in the plasma protein binding of pentazocine in neurosurgical patients than in normal control subjects, in spite of protein concentrations within normal range for both groups.

3. In normal individuals there were only small inter-individual variations in the plasma protein binding of pentazocine, amobarbital, pentobarbital and diphenylhydantoin. Within this small range, the degree of binding of pentobarbital was correlated to that of amobarbital and diphenylhydantoin suggesting a similarity of binding sites.

4. The uremic patient group showed a decreased degree of plasma protein binding of pentobarbital as well as of diphenylhydantoin. The fractions bound in plasma were correlated to the serum creatinine concentration, to the serum albumin concentration and to the hematocrit value.

5. Appreciable proportions of pentazocine, amobarbital, pentobarbital and diphenylhydantoin were found to be localized to the blood cells of the whole blood in healthy volunteers. Uremic patients, too, carry significant proportions of pentobarbital and diphenylhydantoin in the blood cells of the whole blood, in spite of reduced hematocrit values.

6. The ratio between concentration in blood cells : concentration in plasma water of the uremic whole blood was not significantly different from the ratio in normal whole blood, indicating that uremia does not change the intrinsic affinity of the blood cells for the drugs.

7. Salicylic acid displaced pentobarbital and diphenylhydantoin from plasma protein binding sites, but high levels of salicylic acid did not influence the ratio between concentration in blood cells : concentration in aqueous phase for the two drugs.

8. The binding of diphenylhydantoin to the blood cells was the same at 37° C and 25° C, in contrast to the plasma protein binding and these circumstances can cause some analytical error resulting in too high plasma concentration values when whole blood is withdrawn at 37° C but equilibrated and separated at lower temperatures.

9. The two compartment open body model could satisfactorily describe pentazocine pharmacokinetics following intravenous injection. Data suggested high hepatic clearance of the drug which might result in decreased bioavailability of a completely absorbed oral preparation. Pentazocine levels in the cerebrospinal fluid (csf) increased rapidly following the injection and the csf / plasma concentration ratio during the β-phase agreed well with values for unbound / total pentazocine in plasma.

10. Pentobarbital plasma levels following intravenous injection could properly be interpretated with aid of the two compartment model. The body weight appeared to influence the peripheral to central compartment distribution ratio, the drug being further distributed to the peripheral compartment with increased body weight. In the central compartment the drug was mainly located in the associated fluid and only minor fractions of the drug were distributed to the plasma proteins and the blood cells. Pentobarbital had low hepatic clearance and the biological availability of the drug following oral administration was high (94 per cent) in agreement with the calculated bioavailability of completely absorbed oral pentobarbital by application of intravenous clearance concepts.

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